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(54) IMPROVEMENTS IN AND RELATING TO A PROCESS FOR PREPARING GLUCOSE-1-PHOSPHATE

(71) We, HAYASHIBA COMPANY, a Body Corporate, organised and existing under the laws of Japan, of 198, Shimonishii, Okayama-shi, Okayama, Japan, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a process for preparing glucose - 1 - phosphate, and in particular to a process for preparing glucose - 1 - phosphate which comprises using α - 1,6 - glucosidase to decompose starch or starch decomposed substances such as liquefied starch and soluble starch and then using phosphorylating enzymes to produce the glucose - 1 - phosphate.

Glucose - 1 - phosphate was recognized by Cori et al. as a primary intermediate product in glycolysis in vivo, called a Cori ester, and is an interesting compound from biochemical standpoint. There are two processes for manufacturing glucose - 1 - phosphate (referred to as G - 1 - P hereinafter); one being a chemical synthesis and the other being enzymatic. In the chemical process, since glucose is a polyhydric alcohol, it is difficult to produce a specific α - D - glucose - 1 - phosphoric ester by a simple process alone; the yield is poor and the product impure.

In a known process using enzymes, glucogen, starch, or starch decomposed substances are treated with a phosphorylating enzyme (phosphorylase), which may be obtained from a plant or an animal, to decompose a molecule of glucose to the phosphoric ester (G - 1 - P) from the non - reductive terminal of polysaccharide, similar to the reaction *in vivo*. Phosphorylase is well known and there have been many reports on the decomposition of readily available starch or soluble starch by phosphorylase. According to Katz's report in the decomposition of starch by the phosphorylase of the common potato which is said to be most available), amylose is decomposed 100% and amylopectin is decomposed 60%. According to Swanson, glycogen is decomposed 20% and amylopectin is decomposed 40% with the formation of limit dextrins, and thus decomposition is stopped before completion. Recently, there have been the McCready-Hassid method and the Ashby method based on the decomposition of soluble starch. According to these processes, starch is treated with phosphorylase of a common potato to produce G - 1 - P in phosphate buffer solution, and the phosphoric acid in the solution is removed as magnesium-ammonium complex salt; cations are then removed by a strongly acidic ion exchange resin and a weak basic ion exchange resin is allowed to absorb G - 1 - P: then, the G - 1 - P is eluted from potassium hydroxide solution and a 50% alcohol solution is prepared to crystallize its potassium salt. In these processes, however, such substances as consist of only α - 1,4 - glucoside bonds, for instance amylose, can be decomposed by phosphorolysis, but as to substances comprising more than 80% amylopectin containing many α - 1,6 - glucoside bonds such as the common starch α - 1,6 bond cannot be phosphorylated, and decomposition stops before the 1,6 bond: therefore, a glucosidase was required which would decompose α - 1,6 - glucoside bonds but such enzymes rarely exist in plants, and thus β - limit dextrin for example remains. Consequently, the yield remains lower than 50—60% and is inconsistent.

According to the present invention there is provided a process for preparing glucose - 1 - phosphate comprising decomposing a raw material chosen from glycogen, starch, liquefied starch and soluble starch with α - 1,6 - glucosidase and reacting the resultant decomposed products with phosphorylase.

In one embodiment the α - 1,6 - glucosidase is added to the reaction mixture during the phosphorylation of the raw material.

[*Price 25p*]

Suitably the α - 1,6 - glucosidase is the enzyme produced by a culturing at least one strain chosen from the genera *Pseudomonas, Escherichia, Acrobacter, Nocardia, Streptomyces, Actinomyces, Micromonospora, Thermonospora* and *Lactobacillus.*

In particular the α - 1,6 - glucosidase can be obtained by culturing strains such as:

Escherichia intermedia	ATCC	21073
Pseudomonas amyloderamosa	ATCC	21216
Streptomyces diastatochromogenes	IFO	3337
Actinomyces globisporus	IFO	12208
Nocardia asteroides	IFO	3384
Micromonospora melanosporea	IFO	12515
Thermonospora viridis	IFO	12207
Actinoplanes philippinensis	KCC ACT—	0001
Streptosporangium roseum	KCC ACT—	0005
Agrobacterium tumefaciens	IFO	3085
Azotobacter indicus	IFO	3744
Bacillus cereus	IFO	3057
Erwinia aroideae	IFO	3057
Micrococcus lysodeikticus	IFO	3333
Mycobacterium phlei	IFO	3158
Sarcina albida	IFM	1012
Serratia indica	IFO	3759
Staphylococcus aureus	IFO	3061
Lactobacillus brevis	IFO	3345
Leucomostoc citrovorum	ATCC	8081
Pediococcus acidilactici	IFO	3884
Streptococcus faccalis	IFO	3128
Acrobacter aerogenes	ATCC	8724
Corynebacterium sepedonicum	IFO	3306
Aeromonas hydrophila	IFO	3820
Flavobacterium esteroaromaticum	IFO	3751
Acetobacter suboxydans	IFO	3130
Vibrio metschnikovii	IAM	1039
Enterobacter aerogenes	ATCC	8724

The reaction is reversible, and it has been found that molecules of low molecular weight in the decomposed material act as primers for reverse synthesis; thus, the decomposition ratio should be kept at the minimum. The starch raw materials may be cereal or root or tuber starches, for instance, corn starch, waxy maize starch, amylomaize starch, common potato starch, sweet potato starch, tapioca starch, and glutinous rice starch.

Suitably the raw material is soluble starch or liquefied starch.

The starch may be heated and gelatinized at a temperature higher that 150°C to form starch having a dextrose equivalent (D.E.) of from 1% to 5%, which is then rapidly cooled to a temperature in the range of from 45° to 55°C and α - 1,6 - glucosidase added to decompose the α - 1,6 - bonds and then it is used as the raw material for phosphorylation; or such starch is liquefied at 85° to 95°C, for example 90°C with α - amylase, and after liquefying to a DE of 1 to 5%, cooled quickly and the α - 1,6 - glucosidase then added so as to hydrolyze the α - 1,6 - glucoside bond and phosphorylation is then carried out. In an embodiment of the invention the decomposition of the raw material by α - 1,6 - glucosidase and phosphorylation is carried out simultaneously, and the α - 1,6 - glucosidase may be added to the reaction product during the phosphorylation of the raw material.

Phosphorylase for use in the process of the present invention may be found in a wide range of plants, animals and microorganisms. It is advantageous to use a readily available phosphorylase such as one which needs no coenzymes; it is, therefore, preferable to employ phosphorylate enzymes from plants such as the common potato and sweet potato and phosphorylate enzymes produced by microorganisms such as *Neisseria perflave* and yeast.

The process for decomposing starch with two enzymes, α - 1,6 - glucosidase and phosphorylase, may be carried out as follows: purified starch is at first heated at 160° to 170°C for about 5 minutes in a 10% to 20% suspension at a pH of from 5.5 to 6.0 to gelatinize it to a DE of from 1% to 5%. Then α - 1,6 - glucosidase is added at a pH of from 5 to 6 at 45° to 55°C to hydrolyze the α - 1,6 - bonds contained in amylopectin in starch to convert to a straight chains amylose-type molecule, which is used as a sub-

strate for phosphorylation: or starch is liquefied at 85 to 95°C with α-amylase to produce a liquefied solution of a D.E. of from 1% to 5%, which is reacted with α - 1,6 - glucosidase to obtain the reaction substrates, as described previously. In another procedure, the liquefied or gelatinized starch as described above is diluted and reacted with α - 1,6 - glucosidase and phosphorylase simultaneously. Consequently, by using α - 1,6 - glucosidase, the yield of G - 1 - P can be increased by up to 20% to 30%. This latter process will now be explained in more detail. Phosphate buffer solution at pH 6.8 is added to the dilute solution of starch, and at a high concentration of phosphate, a solution of phosphorylase isolated from common potato is added, simultaneously adding a solution of α - 1,6 - glucosidase, and the mixture is allowed to stand to react at 25°C to 30°C for 2 or 3 days. Then, the residual phosphoric acid is removed with magnesium acetate.

The result is compared with that obtained in a similar procedure but without α - 1,6 - glucosidase; in the reaction solution containing phosphorylase without α - 1,6 - glucosidase, magnesium salts hardly precipitate owing to residual dextrin, and also due to impurities such as insoluble dextrin it is difficult to filter and refine the crystal. The reaction according to the invention progresses to completion, magnesium salts quickly precipitate, and the crystals are readily filtered. After removing the magnesium complex the solution is condensed, and then after adding barium acetate, G - 1 - P is precipitated as the barium salt by adding alcohol. The barium salt thus obtained is dissolved in water, and while cooling dilute sulphuric acid is added to precipitate and remove the barium. The pH of the filtrate is adjusted to 8.3 with potassium hydroxide solution, and made up to a 50% solution in ethanol, which is cooled to precipitate crude crystals of the potassium salt of G - 1 - P.

The reaction product obtained only with phosphorylase produces in this stage considerable dextrin precipitation, which is difficult to separate. After treating the product with α - amylase to make it soluble, proteins are removed with trichloroacetic acid, and the residual solution is made alkaline and cooled to obtain the potassium salt of G - 1 - P. In these operations, the reaction solution not treated with α - 1,6 - glucosidase contains undecomposed dextrin which remains up to the final stage and inhibits the operations: the reaction solution obtained after adding α - 1,6 - glucosidase is clear and readily filtered, and precipitation of dextrin is not detected.

The yield of G - 1 - P is 92% when adding α - 1,6 - glucosidase, and 56% in other cases.

The crystals of G - 1 - P potassium salt are dissolved in water to make an aqueous solution, decolourized with active carbon to refine them, and recrystallized by adding acetone: the product contains 8.20% phosphorus and is almost pure crystals of

$$C_6H_{11}O_5 . PO_4K_2 . 2H_2O.$$

By the process of the present invention G - 1 - P can be economically produced from the commercially available starch; by using α - 1,6 - glucosidase, a yield of G - 1 - P of nearly 100% can be obtained. Further as it is an enzyme reaction, other isomers or 2—3 substitution products are not produced; and due to decomposition by α - 1,6 - glucosidase, limit dextrin does not remain and the liquid is clear; thus filtering and refining may be easily performed.

The invention will now be further described with reference to the following Examples:

Example 1

a) Preparation of phosphorylate:

5 Kg. of common potato were well washed with water and after drying with air, grated into a vessel containing toluene floating in 1M phosphate buffer solution, while cooling at 5°C. The solution was roughly filtered through cloth, and centrifuged with a centrifuge at 3000 r.p.m. for 15 minutes to obtain 2920 ml. of the supernatant fluid. The solution was used in the following phosphorylsis.

b) Phosphorylation:

A liquid prepared by dissolving 75 g. of common potato liquefied starch in 4.1 liter of warm water, 1.46 liter of 1M phosphate buffer solution (pH 6.8), 1.46 liter of the above-described solution of the extracted enzyme, and 100 ml. of toluene were mixed together and divided into two equal portions. To one portion (A) 20 units of α - 1,6 - glucosidase (pululanase) per gram of starch were added and to the other portion (B) α - 1,6 - glucosidase was not added. Each solution was made up to 8.0 liters, and allowed to react at 25°C for 72 hours. After heating at 95°C to inactivate the enzyme, 250 g. of magnesium acetate and ammonia water were added to adjust the pH to 8.3: after allowing the resulting solution to stand for 24 hours, the unreacted inorganic phosphoric acid was removed in the form of its magnesium salt. In this step, in the solution (A) the magnesium salt precipitated quickly and was easily filtered out compared with the solution (B).

The filtrate was condensed under reduced pressure at a temperature lower than 50°C e.g. between (45° and 50°C) to make 4 liters, and then the pH was adjusted again to 8.3 by adding 7% ammonium hydroxide, but precipitation was no longer observed. To this solution, 85 g. of barium acetate and 7 liters

of alcohol were added to precipitate glucose - 1 - phosphate as the barium salt, and after 24 hours, the crystalline product was separated by centrifuging. 1 liter of cold water was added to the crystals thus obtained, and while cooling at 2°C in a salt ice bath, 2N sulphuric acid was added to adjust the pH to 2.0. The precipitate of barium sulphate thus produced was quickly separated by centrifuging and the crystals obtained twice washed with cold pure water; the supernatant liquid was adjusted to pH 8.3 with 25% potassium hydroxide solution, and after adding twice the amount of ethanol, it was allowed to stand in a cold place. The solution (A) crystallized on the first day and the solution (B) on the second day. In the solution (B) undecomposed dextrin precipitated out. The precipitate was filtered off, and each solution (A) and (B) was dissolved in 500 ml. of water adjusted to pH 5.5 with acetic acid, and after adding 200 units of liquefying enzyme (α-amylase), allowed to react at 45°C for 2 hours; the iodine reaction showed slight red. After adding 50 ml. of 50% trichloroacetic acid to each solution, 700 ml. of ethanol were added; the solution was immediately filtered to remove precipitates such as protein. The filtrate was adjusted to pH 8.3 with a potassium hydroxide solution to precipitate crystals. After allowing the solution to stand at 15°C for 48 hours, the crystals obtained by filtration were dried in vacuum to obtain the crystals of potassium glucose - 1 - phosphate. The yield was 73 g. with (A) and 44 g. with (B).

The crystals were dissolved in 500 ml. of water and decolourized at 50°C for 30 minutes after adding 3 g. of water-vapour activated charcoal; an equal amount of acetone was added to the filtrate, and after dissolving at 50°C, the solution was crystallized at 25°C. Then, 600 ml. of acetone were added again, and after allowing the solutions to stand at 15°C for 2 days, the crystals were filtered off. The quantitative determination of phosphorus resulted in 8.3%, near the theoretical value of

$$C_6H_{11}O_5 . PO_4K_2 . 2H_2O$$

and it was recognized to be nearly pure.

Example 2

A 10% suspension of starch at pH 5.5 was gelatinized continuously at 160°C for 15 minutes, and cooled quickly at 55°C; 40 units of α - 1,6 - glucosidase produced from *Nocardia asteroides* IFO 3384 per gram of starch were added to the product and the mixture allowed to react at 45°C for 35 hours. The amylose thus obtained was treated to phosphorolysis by the procedure described for portion B in part (b) of Example 1 and crude crystals of potassium glucose - 1 - phosphate was obtained with the yield of 93%. In this Example, a little dextrin precipitated in the reaction solution, and refining was readily performed.

WHAT WE CLAIM IS:—

1. A process for preparing glucose - 1 - phosphate comprising decomposing a raw material chosen from glycogen, starch, liquefied starch and soluble starch with α - 1,6 - glucosidase and reacting the resultant decomposed products with phosphorylase.

2. A process according to Claim 1, wherein the decomposition of the raw material by α - 1,6 - glucosidase and phosphorylation is carried out simultaneously.

3. A process according to Claim 2, wherein the α - 1,6 - glucosidase is added to the reaction mixture during the phosporylation of the raw material.

4. A process according to any of Claims 1 to 3, wherein the α - 1,6 - glucosidase is the enzyme produced by culturing at least one strain chosen from the genera *Pseudomonas, Escherichia, Aerobacter, Nocardia, Streptomyces, Actinomyces, Micromonospora, Thermonospora* and *Lactobacillus*.

5. A process for preparing glucose - 1 - phosphate according to Claim 1, as herein described.

6. A process for preparing glucose - 1 - phosphate according to Claim 1, as herein described with reference to Example 1.

7. A process for preparing glucose - 1 - phosphate, as herein described with reference to Example 2.

8. Glucose - 1 - phosphate whenever prepared by the process of any of Claims 1 to 7.

PAGE, WHITE & FARRER,
Chartered Patent Agents,
27 Chancery Lane,
London, WC2A 1NT.
Agents for the Applicants.

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